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Decreased Brain pH as a Shared Endophenotype of Psychiatric Disorders

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Although the brains of patients with schizophrenia and bipolar disorder exhibit decreased brain pH relative to those of healthy controls upon postmortem examination, it remains controversial whether this finding reflects a primary feature of the diseases or is a result of confounding factors such as medication and agonal state. To date, systematic investigation of brain pH has not been undertaken using animal models that can be studied without confounds inherent in human studies. In the present study, we first reevaluated the pH of the postmortem brains of patients with schizophrenia and bipolar disorder by conducting a meta-analysis of existing data sets from 10 studies. We then measured pH, lactate levels, and related metabolite levels in brain homogenates from five neurodevelopmental mouse models of psychiatric disorders, including schizophrenia, bipolar disorder, and autism spectrum disorder. All mice were drug naive with the same agonal state, postmortem interval, and age within each strain. Our meta-analysis revealed that brain pH was significantly lower in patients with schizophrenia and bipolar disorder than in control participants, even when a few potential confounding factors (postmortem interval, age, and history of antipsychotic use) were considered. In animal experiments, we observed significantly lower pH and higher lactate levels in the brains of model mice relative to controls, as well as a significant negative correlation between pH and lactate levels. Our findings suggest that lower pH associated with increased lactate levels is not a mere artifact, but rather implicated in the underlying pathophysiology of schizophrenia and bipolar disorder.

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INTRODUCTION

Schizophrenia, bipolar disorder, and autism spectrum disorder (ASD) are highly heritable psychiatric conditions, with clinical features transcending diagnostic categories (Hyman, 2010; Insel *et al*, 2010). Accumulating evidence indicates that some genetic influences (Carroll and Owen, 2009; Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013a, b; Lotan *et al*, 2014), gene expression abnormalities (Ellis *et al*, 2016; Shao and Vawter, 2008), and neuronal dysfunctions (Goodkind *et al*, 2015; Yahata *et al*, 2016) associated with these conditions overlap, suggesting a common underlying biological basis. However, the shared neurobiological alterations among the three conditions remain largely unknown.

A number of postmortem studies have indicated that pH is decreased in the brains of patients with schizophrenia and bipolar disorder (Guillozet-Bongaarts *et al*, 2014; Halim *et al*, 2008; Iwamoto *et al*, 2005; Lipska *et al*, 2006; Mistry *et al*, 2013; Prabakaran *et al*, 2004; Ryan *et al*, 2006; Shao and Vawter, 2008; Sun *et al*, 2006; Torrey *et al*, 2005). Decreased brain pH has also been observed in patients with ASD (Young *et al*, 2011). In general, pH balance is considered critical for maintaining optimal health, and low pH has been associated with a number of somatic disorders (Kraut and Madias, 2014; Narins and Emmett, 1980; Posner and Plum, 1967). Therefore, it is reasonable to assume that lower pH may exert a negative impact on brain function and play a key role in the pathogenesis of various psychiatric disorders. However, lower brain pH has largely been considered as an artifact rather than a pathophysiology of such disorders. Animal studies have indicated that chronic treatment with antipsychotics may affect brain pH by increasing lactate levels (Halim *et al*, 2008), and most patients with these disorders receive chronic antipsychotic treatment throughout their lives. In addition, the agonal state experienced

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before death decreases brain pH (Li *et al*, 2004; Tomita *et al*, 2004; Vawter *et al*, 2006), and this state may differ between patients with psychiatric disorders and controls. In human postmortem studies, it is technically difficult to exclude such confounding factors and to determine whether decreased pH and increased lactate levels are indeed artifacts.

In the present study, we first confirmed that patients with schizophrenia and bipolar disorder exhibit lower postmortem brain pH by conducting a meta-analysis of publicly available data sets. We then measured brain pH in multiple mouse models of psychiatric disorders, which are devoid of such confounding factors, in order to test the hypothesis that decreased brain pH is a pathophysiological manifestation/endophenotype of these disorders rather than a mere artifact. We also measured lactate levels, increases in which have frequently been linked to decreased pH in the brains of patients with psychiatric disorders (Halim *et al*, 2008; Prabakaran *et al*, 2004; Stork and Renshaw, 2005). To our knowledge, the present study is the first to systematically evaluate pH and lactate levels in mouse models of psychiatric disorders that eliminate the confounds inherent in human studies.

We focused on mouse models of psychiatric disorders reported to exhibit neurodevelopmental abnormalities in the brain, a part of which stay at a pseudo-immature state (Hagihara *et al*, 2013). Specifically, we measured pH, lactate, and related metabolite levels in the postmortem brains of the following mouse models: *schnurri-2* (*Shn2*) knockout (KO) mice (Takao *et al*, 2013), forebrain-specific *calcineurin* (*Cn*) KO mice (Cottrell *et al*, 2013; Miyakawa *et al*, 2003; Suh *et al*, 2013; Zeng *et al*, 2001), and *neurogranin* (*Nrgn*) KO mice (Huang *et al*, 2006; Huang and Huang, 2012; Pak *et al*, 2000) as a model of schizophrenia; mice with heterozygous knockout of the calcium/calmodulin-dependent protein kinase II *a* (*Camk2a* HKO mice) (Hagihara *et al*, 2016; Yamasaki *et al*, 2008) as a model of bipolar disorder; and mice with heterozygous knockout of the long isoform of chromodomain helicase DNA-binding protein 8 (*Chd8* HKO mice) (Katayama *et al*, 2016) as a model of ASD. These mouse strains are characterized by mutations in genes implicated in the respective disorders and exhibit molecular and behavioral abnormalities relevant to each condition, indicating good construct and face validities, respectively (as described in detail in the Supplementary Materials and Methods).

MATERIALS AND METHODS

Human Data

We obtained pH data of healthy individuals and patients with schizophrenia and bipolar disorder from the Stanley Medical Research Institute (SMRI) database (<https://www.stanleygenomics.org>). Duplicate data among studies in the database were eliminated. In addition, we comprehensively searched the National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO) database for studies reporting individual pH data using the following terms: schizophrenia, bipolar disorder, autism. We also used pH data for healthy individuals and patients with schizophrenia obtained from the New South Wales Brain Tissue Resource Centre schizophrenia cohort (NSWBTRC-SC) (Fillman *et al*,

2013), and from a study by Dean *et al* (2016). Altogether, 10 publicly available data sets were utilized in the present study (Supplementary Table S1): five schizophrenia data sets (GSE17612, GSE21935, GSE21138, NSWBTRC-SC (Fillman *et al*, 2013), and Dean *et al*, 2016)), one bipolar disorder data set (GSE5392), and four combined schizophrenia and bipolar disorder data sets (SMRI Collection A, SMRI Collection C, GSE35977, GSE53987). In addition, we used data regarding postmortem interval and age from these data sets, as well as data regarding medication from SMRI Collection A, SMRI Collection C, Dean *et al*, 2016, and GSE5392.

Animals

We measured pH, lactate, and related metabolite levels in *Shn2* KO mice (Takao *et al*, 2013) ($n=5$, 6 (controls, mutants)), *Cn* KO mice (Cottrell *et al*, 2013; Miyakawa *et al*, 2003; Suh *et al*, 2013; Zeng *et al*, 2001) ($n=6$, 5), *Nrgn* KO mice (Huang *et al*, 2006; Huang and Huang, 2012; Pak *et al*, 2000) ($n=6$, 5), *Camk2a* HKO mice (Hagihara *et al*, 2016; Yamasaki *et al*, 2008) ($n=5$, 5), *Chd8* HKO mice (Katayama *et al*, 2016) ($n=5$, 5), *disrupted-in-schizophrenia 1* (*Disc1*)-*L100P* mutant mice ($n=6$, 6), *Disc1*-Q31L mutant mice ($n=6$, 6) (Shoji *et al*, 2012), voltage-gated calcium channel β -anchoring and -regulatory protein (*Barp*) KO mice (Nakao *et al*, 2015) ($n=10$, 10), and their corresponding control littermates. *Shn2* KO and wild-type control mice were obtained by breeding heterozygotes with a C57BL/6J background and those with a BALB/cA background (Takao *et al*, 2013). All other strains were characterized by a C57BL/6J background. Both male and female mice were used in the present study, as no difference in pH has been observed between sexes (Catts *et al*, 2005). All mice were between 19 and 45 weeks of age, and no significant difference in age was observed between controls and mutants within each strain (*Shn2* KO, 39.3 ± 3.0 weeks, controls (Con), 34.9 ± 1.2 weeks, $P=0.19$; *Cn* KO, 20.6 ± 0.47 weeks, Con, 20.6 ± 0.36 weeks, $P=0.92$; *Nrgn* KO, 32.9 ± 1.2 weeks, Con, 31.0 ± 0.14 weeks, $P=0.12$; *Camk2a* HKO, 33.0 ± 0.052 weeks, Con, 36.2 ± 1.9 weeks, $P=0.13$; *Chd8* HKO, 41.0 weeks, Con, 41.0 weeks; *Disc1*-*L100P* Mut, 29.0 ± 0.41 weeks, Con, 29.0 ± 0.40 weeks, $P=0.97$; *Disc1*-Q31L Mut, 36.1 ± 0.60 weeks, Con, 36.3 ± 0.66 weeks, $P=0.80$; *Barp* KO, 20.3 weeks, Con, 20.3 weeks). All animal experiments were approved by the institutional animal care and use committee of Fujita Health University, based on the Law for the Humane Treatment and Management of Animals and the Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain. Every effort was made to minimize the number of animals used. *Shn2* KO, *Cn* KO, *Nrgn* KO, *Camk2a* HKO, and *Chd8* HKO mice—but not *Disc1*-*L100P* mutant and *Disc1*-Q31L mutant, or *Barp* KO mice—exhibit good construct and face validities for schizophrenia, bipolar disorder, and ASD. Further details are included in the Supplementary Materials and Methods (Supplementary Table S2).

Measurement of pH

Mice were killed via cervical dislocation and decapitation, following which whole brains were removed. The brains were immediately frozen in liquid nitrogen and stored

at -80°C until use. We measured brain pH as previously described (Catts *et al*, 2005; Halim *et al*, 2008). Briefly, mouse brains were homogenized using a tissue homogenizer equipped with a conical pestle in ice-cold distilled H_2O (5 ml per 500 mg of tissue). The pH was measured using a pH meter (LAQUA F-72, Horiba Scientific, Kyoto, Japan) after a three-point calibration at pH 4.0, pH 7.0, and pH 9.0. The pH experiments were performed in triplicate for each sample, following which homogenates were immediately frozen and stored at -80°C until required for further analyses.

Lactate and Glucose Measurements

The concentration of lactate in the brain homogenates was determined using a multi-assay analyzer (GM7 MicroStat; Analox Instruments, London, UK) in accordance with the manufacturer's instructions. In our prior tests using several samples, we loaded 5, 10, and 20 μl of supernatant to the instrument, observing linear, volume-dependent increases in the measured values ($r^2 > 0.99$). Based on these results, we used 20 μl of supernatant for lactate measurements. Similarly, glucose concentrations in 20 μl supernatant samples were determined using a multiassay analyzer following calibration with 10 mmol/ml glucose standard solution. To normalize the effects of differences in genetic background and age among strains, Z-scores for pH and lactate levels were calculated within each strain and used for the correlation analysis.

Pyruvate Measurement

Pyruvate concentrations in 20 μl supernatant samples were determined using a pyruvate assay kit (BioVision, Mountain View, CA). The fluorescence intensities were measured using a microplate reader equipped with a spectrofluorometer (ARVO X, PerkinElmer).

Adenosine Diphosphate/Adenosine Triphosphate (ADP/ATP) Ratio

An ADP/ATP Ratio Assay Kit (BioVision) was used to measure the ADP and ATP concentrations, in accordance with the manufacturer's instructions.

Data Analysis

Human data. We used all data obtained and conducted two-way analyses of variance (ANOVA) and covariance (ANCOVA) for pH, factoring in diagnosis and data set using SAS Studio software version 3.5 (SAS Institute, Cary, NC). Tukey's honest significant difference (HSD) *post hoc* test after ANOVA or ANCOVA was also employed to assess the significance of differences between the mean values of diagnostic groups.

Because of differences in methods used to calculate equivalent doses among the four data sets (fluphenazine *vs* chlorpromazine equivalent), we calculated Z-scores for lifetime antipsychotic use. Z-score transformation—a traditional method of data normalization for direct comparison between different samples and conditions—was applied for each antipsychotic equivalent value and pH value using

individual participant data within each of four data sets, according to the following formula:

$$Z\text{-score} = (\text{value}_P - \text{mean value}_{P1\dots Pn}) / \text{standard deviation}_{P1\dots Pn},$$

where P is any pH and $P1\dots Pn$ represent the aggregate measure of all antipsychotic equivalent or pH values.

Mouse data. Student's t -test was employed to assess the significance of differences between the mean values of controls and mutants in combination with the Bonferroni–Holm correction for repeated measurements. Z-scores for pH and lactate levels were calculated within strains as described above.

Transcriptome Analysis and Bioinformatics Analysis

We used the following mouse brain transcriptome data: frontal cortex and hippocampal dentate gyrus (DG) of *Shn2* KO mice (microarray) (Takao *et al*, 2013), hippocampal DG of *Camk2a* HKO mice (microarray) (Hagihara *et al*, 2009), and whole brains of *Chd8* HKO mice (RNA-sequencing) (Katayama *et al*, 2016). Gene expression patterns in the frontal cortex of *Camk2a* HKO mice ($n=6$, 6) and hippocampal DG of *Cn* KO mice ($n=6$, 6) were analyzed via microarray (Mouse Genome 430 2.0 Array; Affymetrix, Santa Clara, CA), as previously described (Takao *et al*, 2013). Gene expression patterns in the frontal cortex and hippocampal DG of *Nrgn* KO mice ($n=5$, 5) were analyzed via RNA-sequencing using the HiSeq platform in accordance with the manufacturer's instructions (Illumina, San Diego, CA). A total of eight transcriptome data sets were used in the present study. Genes with an absolute fold change > 1.2 and a t -test P -value < 0.05 (mutants *vs* controls; without correction for multiple testing) were imported into the bioinformatics tool BaseSpace (Illumina), with which the gene expression data obtained from different platforms were matched (Hagihara *et al*, 2014). Genes meeting the above criteria are included in Supplementary Table S3. Genes with altered expression in at least four of the eight data sets (yielding 80 features; Supplementary Table S3) were selected based on the criteria of the BaseSpace tool and assessed for enrichment in biological themes using the DAVID functional annotation clustering tool, ADGO, and GOToolBox, in which the default feature listings and algorithm settings were used.

RESULTS

Lower pH in the Postmortem Brains of Patients with Schizophrenia and Bipolar Disorder

We first performed a meta-analysis of postmortem studies regarding brain pH in patients with schizophrenia and bipolar disorder that consisted of nine schizophrenia data sets and five bipolar disorder data sets (Figure 1 and Supplementary Table S1). A two-way ANOVA revealed significant effects of diagnosis ($F_{2,694} = 22.01$, $P = 5.32 \times 10^{-10}$) and data set ($F_{9,694} = 17.93$, $P < 2.00 \times 10^{-16}$), although no interaction was observed between the two factors ($F_{12,694} = 1.70$, $P = 0.063$; Figure 1). The *post hoc* comparisons with Tukey's HSD test indicated that patients with schizophrenia (adjusted $P < 1.0 \times 10^{-7}$) and bipolar disorder (adjusted $P = 9.6 \times 10^{-6}$)

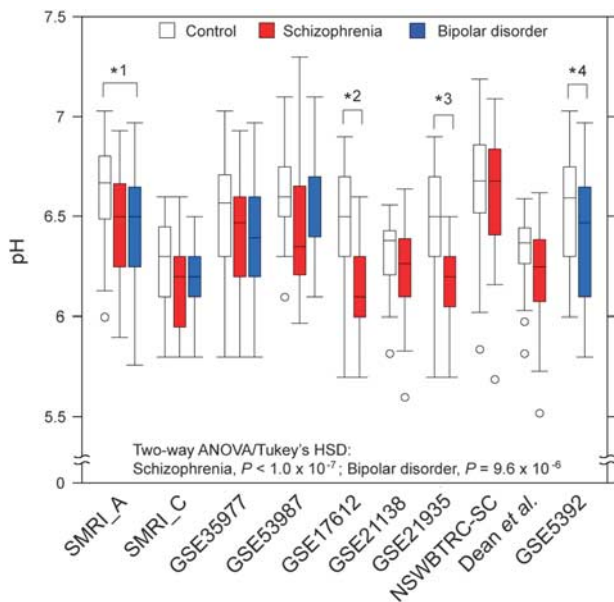


Figure 1 Lower pH in the postmortem brains of patients with schizophrenia and bipolar disorder. Box plot of brain pH in control participants (white box), patients with schizophrenia (red box), and patients with bipolar disorder (blue box). The boxes represent the interquartile range between first and third quartiles, whereas the whiskers represent the maximum and minimum values, and the circles represent population outliers. *¹ $P=0.020$, *² $P<0.0001$, *³ $P=0.0001$, *⁴ $P=0.027$; ANOVA/Tukey's *post hoc* test within each data set.

exhibited lower brain pH than healthy control. However, no significant difference in brain pH was observed between patients with schizophrenia and bipolar disorder (adjusted $P=0.88$). Similar results were obtained with Z-score-transformed data: pH was significantly lower in patients with schizophrenia (adjusted $P<1.0\times 10^{-7}$) and bipolar disorder (adjusted $P=1.59\times 10^{-5}$) than in healthy controls, and no significant difference was observed between patients with schizophrenia and bipolar disorder (adjusted $P=0.85$) (Supplementary Figure S1).

We observed no significant interactions between diagnosis and postmortem interval/age in either schizophrenia (postmortem interval: $F_{1,485}=2.56$, $P=0.11$; age: $F_{1,485}=0.93$, $P=0.34$) or bipolar disorder data sets (postmortem interval: $F_{1,295}=0.63$, $P=0.43$; age: $F_{1,295}=1.07$, $P=0.30$). Hence, we performed correlation analyses between pH and such variables using the combined data of patients and controls to test their effects on pH. In schizophrenia data sets, significant correlations were observed between pH values and age ($r=-0.17$, $P=0.00013$), but not between pH and postmortem interval ($r=0.064$, $P=0.14$). In bipolar disorder data sets, no significant correlations were observed between pH and postmortem interval ($r=0.072$, $P=0.20$), or between pH and age ($r=0.032$, $P=0.57$) (Supplementary Figure S2). Therefore, we performed an ANCOVA (factors: diagnosis and data set) on schizophrenia samples with age as a covariate that revealed a significant main effect of diagnosis ($F_{1,485}=6.16$, $P=0.013$). However, no significant interaction effects were observed for these factors ($F_{8,485}=0.35$, $P=0.95$). In bipolar disorder data sets, as no significant correlations were observed between pH and potential confounding factors, we performed an ANOVA to examine

the effect of diagnosis on pH. Our analysis revealed a significant main effect of diagnosis ($F_{1,305}=9.11$, $P=0.0028$), although no significant interaction effects were observed ($F_{4,305}=0.68$, $P=0.60$). The *post hoc* analyses revealed that pH was lower in the brains of patients with schizophrenia ($P<0.0001$) and bipolar disorder ($P=0.0028$) than in those of healthy controls. Furthermore, Z-score-based meta-analysis of six data sets revealed no significant correlation between pH and lifetime use of antipsychotics ($r=-0.13$, $P=0.094$; Supplementary Figure S3), suggesting that antipsychotic treatment is not a major contributing factor affecting pH in the postmortem brains of patients with schizophrenia and bipolar disorder. Collectively, the results of our meta-analysis support the notion that lower brain pH is a pathological feature of schizophrenia and bipolar disorder rather than an artifact.

Lower pH and Increased Lactate Levels in the Postmortem Brain of Mouse Models of Schizophrenia, Bipolar Disorder, and ASD

The potential confounding factors identified in previous studies (Halim *et al*, 2008; Tomita *et al*, 2004) are beyond the investigator's control in postmortem studies of the human brain. We therefore measured pH and lactate levels in the brains of mouse models of schizophrenia (*Shn2* KO, *Cn* KO, *Nrgn* KO mice), bipolar disorder (*Camk2a* HKO mice), and ASD (*Chd8* HKO mice). All mice used were drug naive and killed via cervical dislocation, following which the removed brains were snap-frozen within a few minutes, allowing us to control for differences in agonal state and postmortem interval differences. Brain pH was significantly lower in all five mutant strains than in the corresponding controls (*Shn2* KO, 7.17 ± 0.0060 , Con, 7.20 ± 0.0056 , $P=0.017$; *Cn* KO, 7.08 ± 0.0057 , Con, 7.13 ± 0.0080 , $P=0.0055$; *Nrgn* KO, 7.10 ± 0.017 , Con, 7.16 ± 0.0080 , $P=0.017$; *Camk2a* HKO, 7.14 ± 0.0093 , Con, 7.21 ± 0.0090 , $P=0.0055$; *Chd8* HKO, 7.08 ± 0.0066 , Con, 7.12 ± 0.0031 , $P=0.0040$; Figure 2a).

Significantly higher levels of lactate were observed in the postmortem brains of all mutant mouse strains than in the corresponding controls (*Shn2* KO, 2.98 ± 0.080 mM, Con, 2.55 ± 0.076 mM, $P=0.015$; *Cn* KO, 3.24 ± 0.051 mM, Con, 2.90 ± 0.073 mM, $P=0.015$; *Nrgn* KO, 2.98 ± 0.11 mM, Con, 2.58 ± 0.054 mM, $P=0.015$; *Camk2a* HKO, 2.86 ± 0.024 mM, Con, 2.58 ± 0.037 mM, $P=0.0012$; *Chd8* HKO, 3.04 ± 0.081 mM, Con, 2.58 ± 0.086 mM, $P=0.015$; Figure 2b). Z-score-based analysis revealed a significant negative correlation between pH and lactate levels (Pearson's $r=-0.65$, $P=1.19\times 10^{-7}$; Figure 2c). In addition, lactate levels exhibited a significant negative correlation with age in the control group, but not in the mutant group (Supplementary Figure S4). However, there were no significant differences in age between controls and mutants of each strain (see the Materials and Methods). No significant correlations between age and pH were observed in either the mutant or control groups (Supplementary Figure S4).

Disc1-L100P mutant, *Disc1-Q31L* mutant, and *Barp* KO mice do not exhibit behavioral phenotypes associated with psychiatric disorders (Nakao *et al*, 2015; Shoji *et al*, 2012) (Supplementary Table S2). Unlike other model mice, mice of these three lines exhibited no changes in brain pH or lactate levels relative to those observed in the corresponding

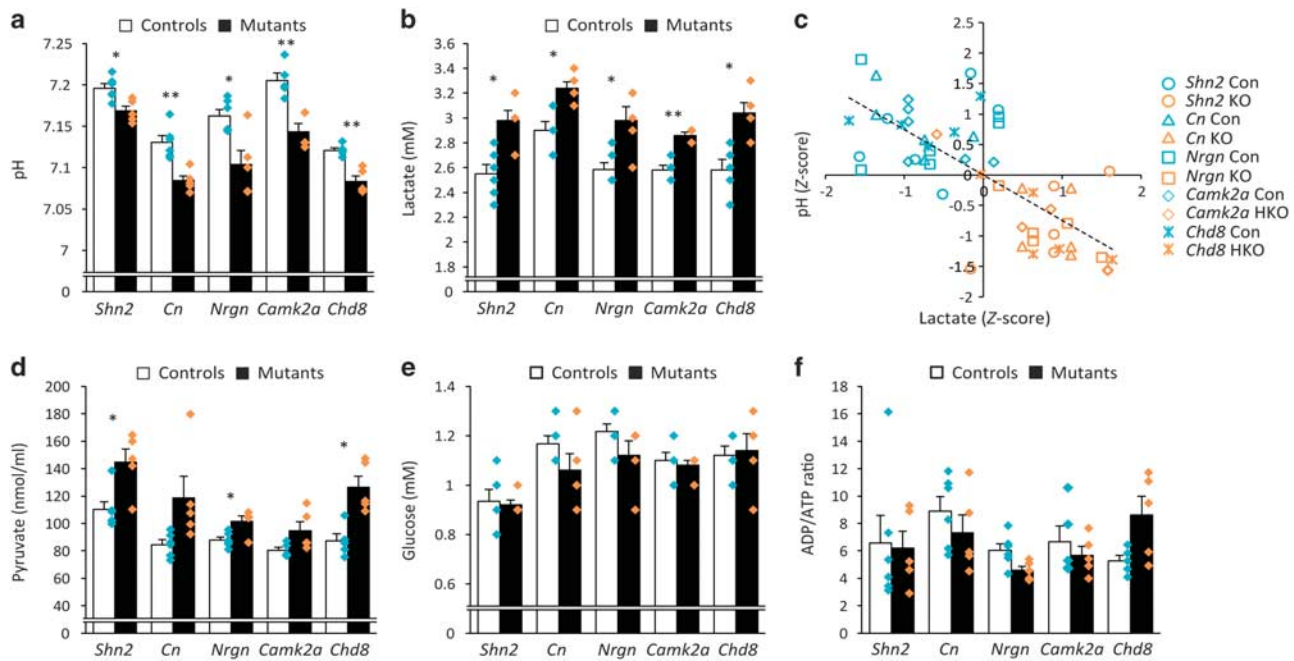


Figure 2 Lower pH and increased lactate levels in the brains of mouse models of psychiatric disorders. Bar graphs of pH (a), lactate levels (b), pyruvate levels (d), glucose levels (e), and ADP/ATP ratio (f) in the brains of *Shn2* KO, *Cn* KO, *Nrgn* KO, *Camk2a* HKO, and *Chd8* HKO mice and their corresponding controls (mean \pm SEM). Each plot represents individual mouse values. (c) Scatter plot showing correlations between pH and lactate levels in the mouse brain. Asterisks indicate statistically significant differences between controls and mutants after Bonferroni–Holm correction (* $P < 0.05$, ** $P < 0.01$). ADP, adenosine diphosphate; ATP, adenosine triphosphate.

controls (Supplementary Figure S5). These results suggest that genetic alterations in general do not necessarily cause changes in pH and lactate levels in the mouse brain.

Lactate is formed from pyruvate during glycolysis. We therefore measured pyruvate levels in model mouse brains, observing that levels were significantly increased in *Shn2* KO ($P = 0.042$), *Nrgn* KO ($P = 0.042$), and *Chd8* HKO mice ($P = 0.042$). *Cn* KO ($P = 0.092$) and *Camk2a* HKO mice ($P = 0.092$) exhibited a trend toward increased pyruvate levels, although this trend did not survive Bonferroni–Holm correction (Figure 2d). Glucose levels remained unchanged in mutant mice relative to controls (Figure 2e), suggesting that glucose supply/demand ratio in the brain may be comparable in these mouse models. In addition, no significant differences in ADP/ATP ratio were observed in these model mice following Bonferroni–Holm correction (Figure 2f), suggesting no alteration of energy consumption ratio in the brains of model mice.

We then analyzed transcriptome data (Supplementary Table S3) in order to investigate the potential underlying molecular mechanisms of increased lactate levels in mutant mouse brains. Transcriptome data from five mouse strains revealed an enrichment in Wnt- and epidermal growth factor (EGF)-related pathways when analyzed using DAVID software (Supplementary Table S4). Enrichment in Wnt-related pathways was replicated in analyses performed using other bioinformatics tools (ADGO and GOTOBox) using different statistical methods (Supplementary Table S4).

As lactate is produced via glycolytic pathways in astrocytes in the brain (Demetrius and Simon, 2012), we analyzed the transcriptome data of model mice with particular focus on glycolysis-related genes (Gene Ontology Consortium database) as well as those related to pyruvate metabolism. The

results of the targeted gene expression analyses suggest that elevated glycolysis and pyruvate metabolism shifting toward lactate synthesis occur in the brains of model mice, especially in *Shn2* KO and *Camk2a* HKO mice (Supplementary Table S5 and Supplementary Figure S6).

DISCUSSION

In the present study, our meta-analysis confirmed that the brains of patients with schizophrenia and bipolar disorder exhibit lower pH than healthy controls upon postmortem examination. Lower pH was also observed in five different mouse models of psychiatric disorders, all of which were drug naive and controlled for other potential confounding factors, such as agonal state and postmortem interval. We also observed increased lactate levels in the brains of model mice, as well as a highly significant negative correlation between pH and lactate levels, consistent with the findings of previous human postmortem studies (Halim et al, 2008). These results suggest that lower pH and increased lactate levels are implicated in the underlying pathophysiology of the diseases rather than mere artifacts.

Our meta-analyses indicated that brain pH is decreased in patients with schizophrenia and bipolar disorder when postmortem interval, age, and antipsychotic use are regarded as potential covariates of brain pH. There are, however, still limitations regarding the covariates in the present study. One major limitation involves the lack of data regarding agonal state, as previous studies have reported that individuals who experience prolonged agonal states exhibit lower brain pH (Mexal et al, 2006; Tomita et al, 2004). These findings suggest that agonal state may be a potential confounding factor for postmortem brain pH. As such, studies using

animal models are necessary to validate the findings from human postmortem studies. However, as we cannot rule out the possibility that agonal states were altered in our model mice, further studies should examine pH and lactate levels in the brains of animals with short, moderate, and prolonged agonal states.

In addition to these limitations, other secondary factors cannot be ruled out in animal studies. For example, a recent study using *Tbx1* mutant mice (ASD model mice) revealed that a genetic risk factor can influence maternal care via the phenotype of the risk carrier (Takahashi *et al*, 2016). Such secondary environmental factors during childhood may thus affect brain pH in mice. Increased locomotor activity may alter oxygen levels in the brains, particularly in *Shn2* KO, *Cn* KO, *Nrgn* KO, and *Camk2a* HKO mice (Supplementary Table S2). Considering that blood oxygen levels are positively correlated with brain lactate levels (Bednařík *et al*, 2015), oxygen levels may also represent a potential confounding factor in the measurement of brain lactate levels. Moreover, the balance between blood and brain cells may differ between the brain homogenates of control and mutant mice. As lactate concentrations in the blood are two- to three-fold higher than those in the mouse brain (extracellular compartment) (Béland-Millar *et al*, 2017), such differences may represent an additional confounding factor. Although differences in genetic background are also of concern when making comparisons between different mouse strains (Wolfer *et al*, 2002), we utilized over nine backcrossings in addition to littermates within each strain. Thus, it is unlikely that differences in pH and lactate levels between control and mutant mice within each strain reflect differences in genetic background.

We observed no significant differences in pH and lactate levels in the brain of two lines of *Disc1* mutant mice as compared with corresponding controls. *DISC1* has been implicated in the genetic etiology of schizophrenia (Brandon and Sawa, 2011; Ishizuka *et al*, 2006). However, a recent large-scale analysis of copy number variants (CNVs) suggested that *DISC1* may not be a risk factor for the disorder (Marshall *et al*, 2016); the analysis showed that there are 5 deletions and 1 duplication in 21 094 schizophrenia cases and 4 deletions and 2 duplications in 20 227 controls (data deposited at UCSC Genome Browser on Human Mar. 2006, NCBI36/hg18). These results, combined with our previous observations that *Disc1* mutant mice used in the present study did not exhibit behavioral phenotypes associated with schizophrenia (Shoji *et al*, 2012), suggest that these mutant mice may not represent a valid animal model of the disorder.

Increased lactate levels have been observed within certain brain regions in patients with schizophrenia (Halim *et al*, 2008; Prabakaran *et al*, 2004), bipolar disorder (Dager *et al*, 2004; Lan *et al*, 2008), and ASD (Goh *et al*, 2014) in both postmortem and *in vivo* spectroscopic imaging studies. More recent studies have confirmed increased lactate levels in the brains of patients with schizophrenia by postmortem analysis (Dean *et al*, 2016) and *in vivo* analysis using 7-Tesla magnetic resonance spectroscopy (MRS) (Rowland *et al*, 2016). Additional studies have revealed that pyruvate levels are increased whereas glucose levels remain unchanged in the postmortem brains of patients with schizophrenia (Dean *et al*, 2016). Our findings in mouse models are

substantially consistent with the evidence obtained from previous human studies.

In our animal experiments, control baselines of pH and lactate levels varied among strains (Figures 2a and b). Such variations may have been due to differences in age, as we observed a significant negative correlation between lactate levels and age in control mice; lactate levels increased as mouse age decreased (Supplementary Figure S4). These findings align with the neurodevelopmental hypothesis of schizophrenia. Accumulating evidence suggests that maturation abnormalities in certain types of brain cells, in which the molecular and physiological properties are similar to those of normal immature cells, represent an endophenotype commonly observed in several neuropsychiatric disorders, including schizophrenia, bipolar disorder, and epilepsy (Hagihara *et al*, 2013; Shin *et al*, 2013; Walton *et al*, 2012). For example, our previous study demonstrated that gene expression patterns in the prefrontal cortex of patients with schizophrenia are strikingly similar to those of typically developing infants (Hagihara *et al*, 2014). Considering the negative correlation between age and lactate levels in control mice of the present study, higher lactate levels in mutant mice may reflect one aspect of maturational abnormalities of the brain. However, additional studies involving detailed time-course analyses of developmental changes in pH and lactate levels are required to verify this hypothesis.

Previous studies have also revealed that brain acidosis influences a number of brain functions, such as anxiety, mood, and cognition (Wemmie, 2011). Acidosis may affect the structure and function of several types of brain cells, including the electrophysiological functioning of GABAergic neurons (Huang *et al*, 2015) and morphological properties of oligodendrocytes (Goldman *et al*, 1989). Alterations in these types of cells have been well documented in the brains of patients with schizophrenia, bipolar disorder, and ASD (Bartzokis, 2005; Nakazawa *et al*, 2012) and may underlie some of the cognitive deficits associated with these disorders. Deficits in GABAergic neurons and oligodendrocytes have also been identified in mouse models of the disorders, including *Shn2* KO mice (Takao *et al*, 2013). Brain acidosis may therefore be associated with deficits in such cell types in schizophrenia, bipolar disorder, and ASD. However, as each genetic alteration may dysregulate the neurochemical balances of downstream molecules that are not functionally relevant to psychiatric disorders, further studies are required to determine whether low pH and increased lactate levels are functionally significant in psychiatric disorders.

A previous study indicated that chronic treatment with antipsychotics increases lactate levels in the rat cerebral cortex (Halim *et al*, 2008), suggesting that such increases may be medication related. The authors of the report, however, found no significant correlation between lactate levels and history of antipsychotic use in the post-mortem brains of patients with schizophrenia. In addition, increased lactate levels have been observed in the anterior cingulate of medication-free patients with bipolar disorder in *in vivo* spectroscopic imaging studies (Dager *et al*, 2004). Furthermore, studies utilizing animal models of psychiatric disorders have identified increased lactate levels in mutant mouse brains *in vivo* (das Neves Duarte *et al*, 2012). In addition, we observed an association between increased lactate levels and decreased pH in the brains of model mice

in the present study, consistent with findings from previous studies on patients with schizophrenia (Halim *et al*, 2008; Prabakaran *et al*, 2004). Decreased brain pH has also been observed in medication-free patients with bipolar disorder (Kato *et al*, 1998). Although it is possible that antipsychotic treatment increases lactate levels and lowers pH in the brain, the aforementioned findings suggest that such changes may occur as primary features of schizophrenia and bipolar disorder.

It should be noted that haploinsufficiency of *Chd8*, a gene associated with ASD, results in lower brain pH and increased lactate levels in mice, as well as molecular and behavioral phenotypes relevant to ASD (Katayama *et al*, 2016). Previous studies have reported that patients with ASD exhibit decreased pH (Young *et al*, 2011) and increased lactate levels (Goh *et al*, 2014) relative to healthy controls, suggesting that our findings in model mice are consistent with those observed in patients with ASD. In addition, *CHD8* has also been implicated in schizophrenia (Kimura *et al*, 2016; McCarthy *et al*, 2014). Therefore, lower pH and increased lactate levels in *Chd8* HKO mice may reflect an aspect of brain pathophysiology in ASD/schizophrenia.

Interestingly, we observed that Wnt- and EGF-related pathways, which are highly implicated in somatic and brain cancers (Nicholas *et al*, 2006), are enriched in the genes whose expressions were altered among the five mutant mouse strains. It is well known that cancer cells display high rates of glycolysis, resulting in high lactate and pyruvate levels, even in normoxia (Lu *et al*, 2002). This phenomenon has been referred to as the Warburg effect. Genes whose expression is known to positively regulate the Warburg effect, such as *Hk2* (Mathupala *et al*, 2009), *Hif1a* (Lu *et al*, 2002), and *Pfkfb3* (Minchenko *et al*, 2002), were increased in the brains of some of mouse models examined in the present study, whereas expression of *Prkaa1*—a negative regulator of the Warburg effect (Faubert *et al*, 2013)—was decreased (Supplementary Table S3). These findings suggest that elevated glycolysis underlies increases in lactate and pyruvate levels in the brains of schizophrenia, bipolar disorder, and ASD model mice. The results of the targeted gene expression analyses conducted in the present study also support this hypothesis. Glycolysis is also stimulated by the uptake of glutamate in astrocytes following neuronal excitation (Pellerin and Magistretti, 1994). Dysregulation of the excitation–inhibition balance has been proposed as a candidate cause of schizophrenia, bipolar disorder, and ASD (Brealy *et al*, 2015; Marín, 2012). A shift in the balance toward excitation would result in increased energy expenditure and may lead to increased glycolysis. Indeed, *Shn2* KO mice exhibit higher glutamate levels in the hippocampus (Takao *et al*, 2013). *In vivo* metabolite measurements have suggested that increased glycolysis also occurs in the brains of patients with schizophrenia (Rowland *et al*, 2016) and bipolar disorder (Dager *et al*, 2004; Stork and Renshaw, 2005), whereas gene ontology analysis of microarray data has suggested that decreased glycolysis occurs in the brains of patients with schizophrenia (Prabakaran *et al*, 2004). Although further studies are required to determine whether alterations in the rate of glycolysis are associated with increased lactate levels and decreased pH, we hypothesize that decreased pH in whole-brain samples was due to increased lactate production driven by hyperactivity within

specific neural circuits (Hagihara *et al*, 2013; Heckers and Konradi, 2015; Lisman *et al*, 2008). Therefore, it would be of interest to investigate pH and lactate levels, as well as glycolysis rate, in the brains of mouse models of other mental disorders in which such hyperactivity has been implicated, such as epilepsy (Seifert and Steinhäuser, 2013), depression (Grace, 2016), and Alzheimer's disease (Busche *et al*, 2012).

Previous studies have indicated that lactate levels in the mouse brain rapidly increase after at least 1 min of decapitation, relative to those observed following *in vivo* fixation via focused microwave irradiation, regarded as a consequence of enhanced glycolysis under oxygen-deprived conditions (Sugiura *et al*, 2014). Although the current findings may differ from those obtained under physiological conditions, they may also reflect functional changes (eg, astrocyte activation) (Huang and Huang, 2012; Takao *et al*, 2013) that represent the main source of lactate production in the brain.

Brain pH is associated with notable changes in gene expression (Catts *et al*, 2005; Iwamoto *et al*, 2005; Mexal *et al*, 2006; Tomita *et al*, 2004) and has hence been considered as a confound for investigating changes in gene expression related to the pathophysiology of psychiatric disorders. Therefore, substantial effort has been made to match tissue pH between patients and controls. Given that lower brain pH is a pathophysiological component of certain conditions, pH-dependent changes in gene expression are of concern when attempting to elucidate the molecular basis of the conditions. Some studies have indicated that gene expression patterns are partially similar across diseases such as schizophrenia, bipolar disorder, and ASD (Ellis *et al*, 2016; Shao and Vawter, 2008). Decreased pH may underlie these similarities in the pattern of gene expression. Thus, pH may be an important factor in the elucidation of molecular alternations in the brains of patients with these psychiatric conditions.

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